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אני, (שם המבקש, מענו ולגבי גוף מאוגד - מקום התאגדותו)
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ששמה הוא
of an invention the title of which is

(בעברית) (Hebrew): שיטה לבידוד ותירבות של מיקרואורגניסמים בלתי ניתנים לגידול בתרבית

(באנגלית) (English): Method for isolating and culturing unculturable microorganisms

מבקש בזאת כי ינתן לי עליה פטנט
hereby apply for a patent to be granted to me in respect thereof.

*בקשת חלוקה - Application of Division	*בקשת פטנט מוסף - Application for Patent Addition	*דרישת דין קדימה Priority Claim		
מבקשת פטנט from Application	*לבקשה/לפטנט to Patent/Appl.	מספר/סימן Number/Mark	תאריך Date	מדינת האגוד Convention Country
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הוגש בעניין filed in ..... case				

חתימת המבקש
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שיטה לבידוד ותירבות של מיקרואורגניסמים בלתי ניתנים לגידול בתרבית

METHOD FOR ISOLATING AND CULTURING UNCULTURABLE MICROORGANISMS

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אוניברסיטת בן גוריון בנגב הרשות למחקר ופיתוח

ממציאים: אריאל קושמרו, שמעונה גרש

FIELD OF THE INVENTION

The present invention relates to a method for isolating and culturing novel "uncultivable" microorganisms.

BACKGROUND OF THE INVENTION

Cultured microorganisms are the most common source of antibiotics and other medicinal agents. However, only a small percentage (less than 0.1%) of the viable bacteria in soil can be cultured on known nutrient media using current techniques such as petri dishes (Handelsman et al. 1998; Amann et al. 1995). The other 99.9% of uncultured/uncultivable microorganisms, with their genetic and biochemical diversity, may emerge as a major source of new natural chemical structures that may be useful for humans for example as drugs.

The exploration of previously uncultured microorganisms for the discovery of new useful natural products is now being carried out in several laboratories. The main approach involves genomics techniques such as the approach designated metagenomics for the analysis of the collective genomes of the microorganisms in the soil community. According to this approach, DNA in large segments is cloned directly from soil into a culturable host and a sequence-based and functional genomic analysis is conducted on it. The intention is the isolation of new signals, new secondary metabolites that might have utility for humans, and the reconstruction of an entire genome of an uncultured organism.

Molecular microbial ecology represents a recent development in research methods. It consists of utilizing techniques of molecular biology to investigate the ecology of microorganisms, and offers new tools to facilitate the detection and identification of microorganisms in the environment.

Molecular microbial ecology allowed the development of tools to address a central dogma of microbial ecology: an inability to cultivate more than a small proportion (0.1–1%) of the bacteria that can be visualized by direct count procedures (Head et al., 1998). Thus, the identification of bacteria by molecular methods represents an indispensable addition to the traditional methods based on the analyses of morphological and physiological characteristics. Among these culture-independent new techniques, the technique based on direct sequencing seems to be the most effective. It consists in sequencing a specific region of the bacterial chromosome, namely the bacterial 16S rDNA region, and in comparing this sequence with known sequences stored in data banks. All microorganisms possess at least one copy of the genes coding for the ribosomal RNA (rRNA), which are indispensable in any cells for the biosynthesis of proteins. Within these genes, the 16S rDNA region is principally used for the determination of the genus and the species of bacteria. By using this approach, it could be determined in many environmental samples the predominance of many different uncultured species. It might be feasible that the yet uncultured types of bacteria might be grown under laboratory conditions if just the right nutrients are found (Amann et al. 1995, Felske et al. 1999).

Recently, Kaeberlein et al. (2002) disclosed a new method for isolating and growing uncultivable microorganisms in pure culture in a simulated natural environment using a diffusion chamber. Microorganisms were separated from intertidal marine sediment particles, serially diluted, mixed with warm agar made with seawater, and placed in the diffusion chamber. The membranes allowed exchange of chemicals between the chamber and the environment but restricted movement of cells. After the first membrane was affixed to the base of the chamber, the agar with microorganisms was poured in, and the top was sealed with another membrane (See Fig.1, Kaeberlein et al. 2002). The diffusion chamber consists of a stainless steel washer (70 mm o.d., 33 mm i.d., 3 mm in thickness)

sandwiched between two 0.03-μm pore-size polycarbonate membranes. The membranes were glued to the washer forming the inner space filled with test microorganisms in semi-solid agar. The sealed chambers were placed on the surface of the sediment collected from the tidal flat and kept in a marine aquarium. Colonies of representative marine microorganisms were isolated in pure culture. These isolates did not grow on artificial media alone but formed colonies in the presence of other microorganisms.

In summary, most microorganisms in the environment have been overlooked as yet due to their resistance to cultivation on artificial media. The cultured microorganisms represent only a small fraction of natural microbial communities and hence the microbial diversity in terms of species richness and species abundance is grossly underestimated. Our understanding of microbial diversity is not represented by the cultured fraction of the microbial community (Wintzingerode et al., 1997).

DESCRIPTION OF THE INVENTION

The present invention relates to a method for isolation and culture of microorganisms suitable for any environmental source.

According to one aspect, the present invention relates to a method for isolating and culturing a previously unculturable microorganism, which comprises:

(i) collecting a sample from an environmental source;

(ii) counting/estimating the number of microorganisms in the sample;

(iii) diluting the sample in an appropriate medium;

(iv) adding a gelating agent such as to entrap one or more microorganisms within a sphere of the gelating agent;

(v) coating the spheres containing the entrapped microorganism(s) with a natural or synthetic polymer

to form a polymeric membrane;

(vi) incubating the coated spheres in the original environment for an appropriate time;

(vii) cutting the spheres and scanning for microorganisms colonies; and

(viii) isolating the microorganisms, and repeating steps (iii) to (vii) until a pure clone of said previously unculturable microorganism is obtained.

The environmental source from which the samples are collected may be any terrestrial, aquatic or marine source such as soil, biofilms, sediments (e.g. coral or other marine sediments, aquifer sediments and the like), waste waters and the like. The sample, suspended in its natural or other appropriate medium, is divided, for example, into 1-ml tubes, and each divisional sample is then subjected to counting/estimation of the number of microorganisms by well-known techniques, for example by DAPI (4',6-diamidino-2-phenylindole) staining of the cells and fluorescence microscopy of the DAPI-stained cells.

In the next step, the samples are diluted as necessary in an appropriate medium. As used herein, an "appropriate medium" is intended to mean a medium compatible with the environment from which the sample has been collected with respect to physico-chemical parameters such as pH, salinity, temperature, oxygen concentration and the like. The medium may be sterile water, sterile saline, sterile water containing suitable ingredients for compatibility with the environmental source and the like. For example, a sample collected from a marine source will have the salinity corresponding to the marine source and the salt concentration will be higher if the sample is originated from the Dead Sea.

The next step consists in the addition of a gelating agent as a matrix to the diluted samples. Any suitable natural, semi-synthetic or synthetic gelating agent may be used such as, but not limited to, alginate and agar. Thus, in one

embodiment, the diluted samples are mixed with warm (40-50°C) autoclaved agar (0.7-2%) such as to entrap preferably one, or more, microorganisms within a sphere of the gelating agent. When the gelating agent is agar, spheres can be obtained by dripping agar droplets into cold mineral oil. The sphere size of 0.1 mm or less up to 5, preferably up to 2-3, mm in diameter, can be determined by the nozzle diameter and dripping rate.

The gelating agent spheres containing the entrapped microorganism(s) are then coated with a natural or synthetic polymer such as, but not limited to, a polysulfone, an alginate, an epoxy resin and the like, such as to form a multilayered membrane. Polysulfone resin of average m.w. 35000, Na-alginate, and epoxy resin such as Epikote 255 have been disclosed before for biomass entrapment for different applications (Ferguson et al., 1989; Blanco et al., 1999).

For coating, the polymer is first dissolved in a suitable solvent (for example, polysulfone may be dissolved in DMF), the dried gelating agent spheres containing the entrapped microorganism are introduced into the polymer solution, and are then transferred into a medium that enables coating of the spheres by several layers of the polymer, thus forming the desired spheres coated by the polymeric membrane. In one embodiment, agar spheres containing one or more microorganisms are immersed into a solution of polysulfone in DMF and transferred to water in order to obtain the desired polymeric coating. The polymeric membrane allows exchange of chemicals between the sphere and the environment but restrict movement of cells.

The next step consists in the incubation of the polymeric coated gelating agent spheres containing one or more microorganisms in the environment from which the original sample has been collected, for an appropriate time. This is the alternative to cultivate such microorganisms that cannot grow in the known growth media for microorganisms. The incubation in the environment can take from days to months.

After the incubation, the spheres are cut and placed on a glass slide and covered with a coverslip, and the entire volume of agar is scanned for microbial colonies at magnification of 400X and 1,000X. The microorganisms are then isolated into pure culture by successive re-inoculation of individual colonies into new spheres followed by incubation in the environment, by repetition of steps (iii) to (vii) as many times as necessary, until a pure clone of the previously unculturable microorganism is obtained.

After incubation in the environment, previously uncultured microorganisms can be isolated and then subjected to molecular biology and genomics techniques, and/or cultured for the production of bioactive materials.

The method of the present invention will allow isolation/identification of new types of microorganisms, such as bacteria, previously considered as unculturable and the establishment of libraries of "uncultivable" microorganisms useful for the drug discovery and biotechnology industries. The method enables exploration of new natural products from previously uncultured microorganisms New genes might be obtained from the previously uncultivable microbial communities, and new biologically active materials such as proteins, enzymes and antibiotics of utility to humans may be discovered.

In another aspect, the present invention relates to a previously uncultured microorganism isolated by the method of the present invention. In a preferred embodiment, the microorganism is a bacterium.

In a further aspect, the present invention relates to a library of previously uncultured microorganisms obtained by the method of the invention and to the use of said library for the discovery of new drugs including, but not being limited, to new antibiotics.

Reference now is made to the Scheme herein just before the claims that shows a flowchart of the method of the invention for isolation and culture of previously unculturable bacteria. Thus, an environmental sample is collected, the

number of bacteria is counted/estimated by microscopic observation as shown in the right side of the figure, the sample is diluted as shown in the right side of the figure in order to entrap approximately one bacterium in one agar sphere, the agar sphere is coated with a polymeric membrane, incubated in the environment, and the bacterium is then isolated, cultured and subjected to molecular biology techniques.

Fig. 1 depicts a schematic drawing of an agar sphere coated with a polymeric membrane.

Fig. 2 is a scanning electron microscopy photograph showing an agar sphere coated with a polysulfone membrane as obtained in the Example hereinafter.

The invention will now be illustrated by the following non-limiting Example.

Example.

An environmental sample was obtained from laboratory scale wastewater bioreactor (waste water from Ramat Hovav Toxic Waste Dumping Site, Negev, Israel) and estimated for microorganism number by DAPI-staining and microscope direct counting (10^8-10^9 cell/mL). The sample was diluted 8- and 9-fold with water in order to entrap approximately one microorganism in one agar sphere.

The diluted samples were mixed with warm (50°C) autoclaved agar (DIFCO) (900 µl agar per 100 µl diluted sample, final concentration 1.5 % agar). Agar spheres of approximately 1-2 mm in diameter containing the entrapped microorganism(s) were formed by dripping droplets of the mixture into cold mineral oil.

A solution of 10% polysulfone of m.w. ca. 35,000 (Sigma-Aldrich, Product No. 42,830-2) in DMF was prepared and used to coat the dried agar spheres containing the entrapped microorganism(s). For this purpose, the agar spheres

were introduced into the polymer solution, and then transferred into water in order to obtain the desired polymeric membrane.

The polysulfone-coated agar spheres containing the entrapped microorganism(s) were then incubated for 3 weeks in a wastewater bioreactor. At the end of the incubation, the agar spheres were cut and placed between a glass slide and a coverslip, and the entire volume of agar was scanned for microbial colonies at magnification of 400X and 1000X.

In the spheres, we could see development of isolated colonies of bacteria and fungi presumably uncultivable, since experiments to grow them in ordinary agar plates were not successful and no bacterial growth was observed.

A further experiment is being carried out now with coral microorganisms taken from coral.

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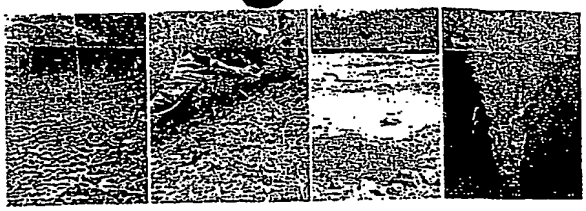
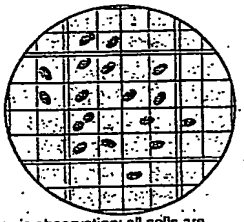
SCHEME

Environmental sample

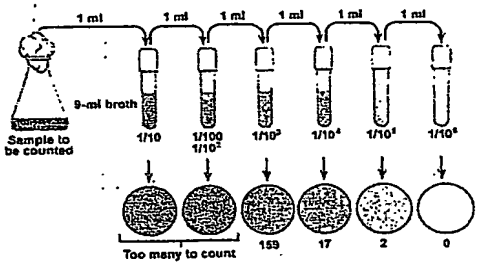


Counting / estimating bacterial #

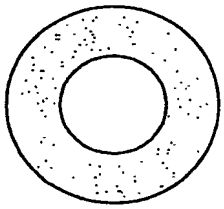


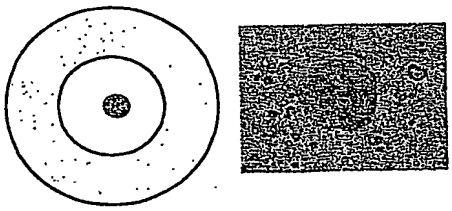
Microscopic observation; all cells are counted in large square: 12 cells (in practice, several squares are counted and the numbers averaged)

Dilution, ~ One bacterium in one agar ball



Coating with polymeric Membrane





Incubating in the environment

Isolation, molecular biology, culturing and production of bioactive materials

CLAIMS:

1. A method for isolating and culturing a previously unculturable microorganism, which comprises:

(i) collecting a sample from an environmental source;

(ii) counting/estimating the number of microorganisms in the sample;

(iii) diluting the sample in an appropriate medium;

(iv) adding a gelating agent such as to entrap one or more microorganisms within a sphere of the gelating agent;

(v) coating the spheres containing the entrapped microorganism(s) with a natural or synthetic polymer to form a polymeric membrane;

(vi) incubating the coated spheres in the original environment for an appropriate time;

(vii) cutting the spheres and scanning for microorganisms colonies; and

(viii) isolating the microorganisms, and repeating steps (iii) to (vii) until a pure clone of said previously unculturable microorganism is obtained.

2. A method according to claim 1 wherein said environmental source is a terrestrial, aquatic or marine source.

3. A method according to claim 1 or 2 wherein said appropriate medium of (iii) is a medium compatible with the environment from which the sample has been collected.

4. A method according to any one of claims 1 to 3 wherein said gelating agent is agar.

5. A method according to any one of claims 1 to 4 wherein said gelating agent sphere of (iv) has a size from 0.1 mm or less to about 5 mm, preferably 1-2 mm in diameter.

6. A method according to any one of claims 1 to 5 wherein said natural or synthetic polymer is a polysulfone, alginate or epoxy resin.

7. A microorganism isolated by the method according to any one of claims 1 to 6.

8. A microorganism according to claim 7 consisting of a bacterium.

9. A library of microorganisms according to claim 7 or 8.

10. Use of a library of microorganisms according to claim 9 for the discovery of new drugs.

11. Use according to claim 10 for the discovery of new antibiotic drugs.

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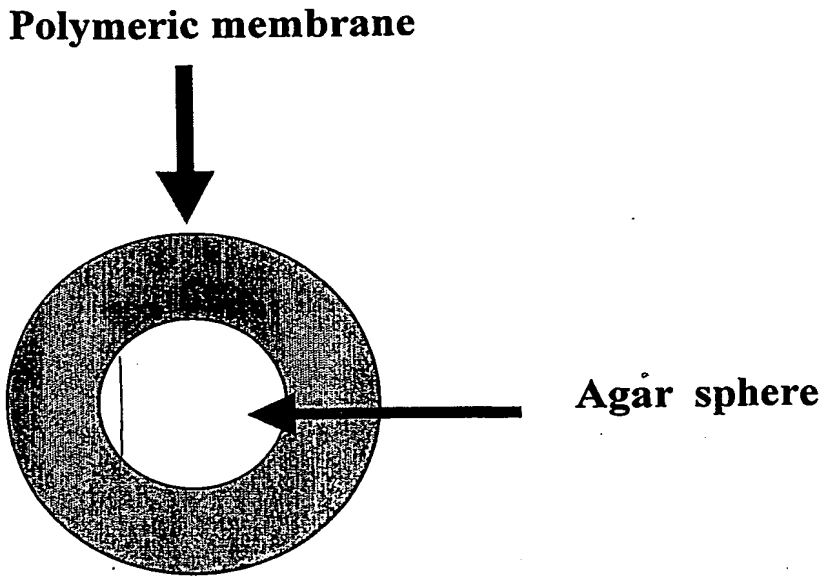

Figure 1

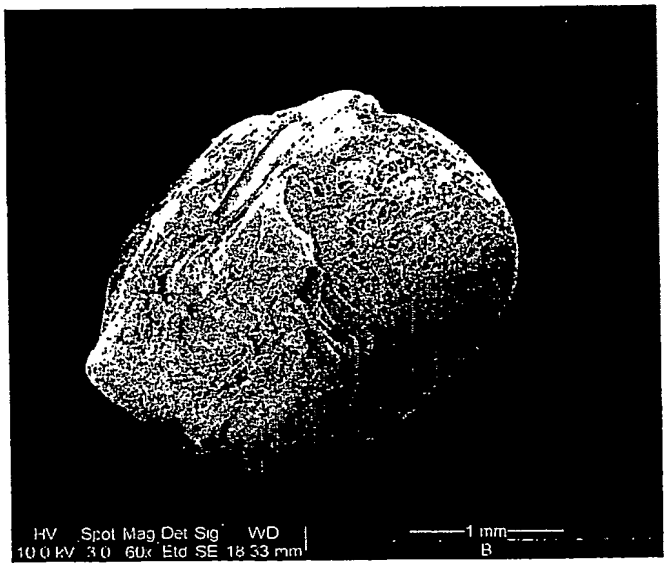

Figure 2